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ROLE OF NEW PODOCYTE-ASSOCIATED PROTEINS IN THE RENAL ULTRAFILTRATION BARRIER

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Cover: confocal microscopy image of a mouse glomerulus (front) and electron microscopy image of a podocyte (back)

Role of new podocyte-associated proteins in the renal ultrafiltration barrier
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

Chronic kidney disease (CKD) is a major health problem and an economical burden affecting people worldwide. The main causes of CKD are diabetes and hypertension and patient numbers keep increasing. In many cases, CKD is progressive leading to end stage renal disease (ESRD), a condition that can be treated only through chronic dialysis or renal transplantation. One of the first clinical signs of CKD is proteinuria due to impaired function of the glomerulus and glomerular podocyte cells. Podocyte damage and eventual loss has been shown to be a common key pathogenic event in many glomerular disease processes leading to CKD and ESRD.
The overall aim of this thesis was to identify and characterize podocyte-specific or enriched proteins in glomerular homeostasis and disease with the goal of discovering potential novel molecular targets for the treatment of CKD.

In study I&II we investigated the role of actin cytoskeleton-associated protein Coro2b. Using confocal and stimulated emission depletion (STED) immunofluorescence microscopy we show that Coro2b is expressed early during glomerulogenesis and only in the podocyte within the kidney cortex of man and mouse. Additionally, STED microscopy revealed localization towards the apical plasma membrane of the podocyte foot processes (FPs). Analysis of Coro2b expression in patient biopsies revealed that it is differentially expressed in diabetic nephropathy (DN) and not in IgA nephropathy (IgAN) or membranous nephropathy. Constitutive and podocyte-specific knock out (KO) of Coro2b in BL6 mice did not affect glomerular development, however targeting the podocyte FP actin cytoskeleton in a protamine sulphate injury model resulted in altered response in the podocyte-specific Coro2b KO mice when compared to their wildtype (wt) littermate controls. In zebrafish embryos, knock-down of Coro2b with morpholinos resulted in podocyte loss/de-differentiation, FP effacement and impaired pronephric filtration barrier function.
In study III we show that orphan GPCR Gprc5a is only expressed in the podocyte within the kidney cortex and downregulated in patients with DN. The constitutive KO of Gprc5a lead to elevated levels of profibrotic markers TGF-β, EGFR and Col1a1, as well as to mesangial matrix expansion and thickening of the glomerular basement membrane in aging mice. Nephropathy, caused by STZ induced diabetes, was more severe in Gprc5a KO mice than in their littermate controls, as shown by higher albuminuria and increased mesangial matrix expansion, presence of totally sclerotic glomeruli and more podocyte FP effacement. In cell culture experiments with immortalized human podocytes, we demonstrated that the overexpression of Gprc5a inhibits the activation or expression of EGFR, TGF-β and SMAD2/3, while the silencing of Gprc5a increases the activation and expression of the same proteins after EGF stimulation.
In study IV we demonstrated that orphan Gprc5b is highly enriched in podocytes and is localized to the apical membrane. Interestingly, Gprc5b is upregulated in DN, IgAN and lupus nephritis. Generation of a podocyte-specific KO mouse line in combination with lipopolysaccharide induced nephropathy, RNA sequencing and cell culture experiments, we showed that Gprc5b activates pro-inflammatory NF-κB/p65 and increases cytokine levels of ccl2 IL-6 an M-CSF1 in podocytes. Gprc5b overexpression also increases the activation/phosphorylation of EGFR and β-catenin, while Gprc5b-KO glomeruli show decreased recruitment of CD45 positive leukocytes.
In study V we demonstrated through expressional profiling that ZFYVE28 is a novel podocyte-enriched protein that localizes to the FPs. Overexpression of ZFYVE28 in cultured human podocytes leads to enhanced EGFR and SMAD2 activation and redistribution. The constitutive and podocyte-

specific KO of ZFYVE28 in BL6 mice showed no developmental or phenotypical changes compared to wt littermates in both health and a model of glomerulonephritis.

Altogether, this thesis provides clinical and biological insights into podocyte organization during health and glomerular disease.

LIST OF SCIENTIFIC PAPERS

I. **Angelina Schwarz**, Katja Möller-Hackbarth, Lwaki Ebarasi, David Unnersjö Jess, Sonia Zambrano, Hans Blom, Annika Wernerson, Mark Lal, Jaakko Patrakka
Coro2b, a podocyte protein downregulated in human diabetic nephropathy, is involved in the development of protamine sulphate-induced foot process effacement
Scientific Reports (2019), vol. 9(1):8888

II. **Angelina Schwarz**, Lwaki Ebarasi, Jaakko Patrakka
Knock down of Coro2ba in zebrafish causes pronephric functional alterations
Manuscript

III. Xiaojie Ma, **Angelina Schwarz**, Sonia Zambrano Sevilla, Anna Levin, Kjell Hultenby, Annika Wernerson, Mark Lal, Jaakko Patrakka
Depletion of Gprc5a Promotes Development of Diabetic Nephropathy
J Am Soc Nephrol (2018), vol. 29(6), p1679-1689

IV. Sonia Zambrano Sevilla, Katja Möller-Hackbarth, Xidan Li, Patricia Rodriguez, Emmanuelle Charrin, **Angelina Schwarz**, Jenny Nyström, Annika Wernerson, Mark Lal, Jaakko Patrakka
Gprc5b modulates inflammatory response in glomerular disease via NF-κB pathway
J Am Soc Nephrol (2019), Jul 8. pii: ASN.2019010089

V. Sonia Zambrano, Patricia Q. Rodriguez, Jing Guo, Katja Möller-Hackbarth, **Angelina Schwarz**, Jaakko Patrakka
FYVE domain-containing protein ZFYVE28 regulates EGFR-signaling in podocytes but is not critical for the function of filtration barrier in mice
Scientific Reports (2018), vol. 8(1), 4712

CONTENTS

LIST OF ABBREVIATIONS

ADR	Adriamycin
AKI	Acute kidney injury
BL6	Black 6 gentic background mouse strain
BRCA1	Breast cancer type 1 susceptibility protein
Ca^{2+}	Calcium cation
cAMP	Cyclic adenosine monophosphate
CAPZ	F-actin capping protein
Cav-1	Caveolin-1
ccl2	Chemokine (C-C motif) ligand 2
CD2AP	CD2-associated protein
cDNA	Complementary DNA
CFL1	Cofilin-1
CHD	Calponin-homology domains
CKD	Chronic kidney disease
CNS	Central nervous system
Col1a1	Collagen 1 alpha 1
Coro2b	Coronin 2B/Coronin-like protein C/Clipin C
CsA	Cyclosporine A
CTGF/CCN2	Connective tissue growth factor
CVD	Cardio vascular disease
DAB	3,3’-diaminobenzidine
DAG	Diacylglycerol
DBD	DNA-binding domain
DKD	Diabetic kidney disease
DN	Diabetic nephropathy
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eGFR	Estimated glomerular filtration rate
EM	Electron microscopy
ERK	Extracellular signal-regulated kinases
ESRD	End stage renal disease
FA	Focal adhesion
FAK	Focal adhesion kinase

FP	Foot process
FSGS	Focal segmental glomerulosclerosis
GBM	Glomerular basement membrane
GDP	Guanine diphosphate
GFB	Glomerular filtration barrier
GFP	Green fluorescent protein
GFR	Glomerular filtration rate
GPCR	G-protein coupled receptor
Gprc5a	G-protein coupled receptor class C group 5 member A/ Retinoic acid-induced protein 3
Gprc5b	G-protein coupled receptor class C group 5 member B/ Retinoic acid-induced protein 2
G-protein	Guanine nucleotide binding protein
GTP	Guanine triphosphate
h	Hour
HBEGF	Heparin-binding EGF-like growth factor
HDAC-1	Histone deacetylase-1
HE	Hematoxylin and eosin
HGF	Hepatocyte growth factor
HRP	Horse radish peroxidase
HS	Heparine sulphate
IgAN	Immunoglobulin A nephropathy
IKK	IκB kinase complex
IL-1β	Interleukin 1-beta
IL-6	Interleukin 6
ILK	Integrin linked kinase
IQAP	Iq-motif containing GTPase-activating protein
I-SMAD	Inhibitory SMAD
Itga3	Integrin alpha 3
Itgb1	Integrin beta 1
kDa	Kilo Dalton
KO	Knock out
LN	Lupus nephhritis
LPS	Bacterial lipo-polysaccharides
MAPK	Mitogen-activated protein kinases
M-CSF1	Macrophage colony-stimulating factor 1
MDCK	Madin-Darby canine kidney

miRNA	MicroRNA
MN	Membranous nephropathy
MN	Membranous nephropathy
MP	Major process
mRNA	Messenger RNA
NCoR	Nuclear receptor corepressor
NEPH1	Nephrin-like protein 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIDDK	National institute of diabetes and digestive and kidney disease
NIH	National institutes of health
NLS	Nuclear localization sequence
NMJ	Neuromuscular junctions
NPXY	Asparagine-proline-any amino acid-tyrosine
NRCF	Neonatal rat cardiac fibroblasts
NSCLC	Non-small cell lung cancer
N-WASP	Neuronal Wiskott–Aldrich syndrome protein
PAS	Periodic acid-Schiff
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGFRβ	Platelet-derived growth factor beta
PFA	Paraformaldehyde
PI3K	posphoinositide 3-kinase
PS	Protamine sulphate
PTU	1-Phenyl-2-thiourea
qPCR	Quantitative polymerase chain reaction
RIPA-buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
R-SMAD	Receptor regulated SMAD
SD	Slit diaphragm
SDS	Sodium dodecyl sulfate
SMAD2/3	Mothers against decapentaplegic homolog 2 and 3
SMS2	Sphingomyelin synthase 2
STED	Stimulated emission depletion
STZ	Streptozotocin

TAD	Transactivation domain
TALEN	Transcription activator-like effector nuclease
TEM	Transmission electron microscopy
TGFBRI&II	TGF-β type I & type II receptor
TGFA	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Tris	Tris(hydroxymethyl)aminomethane
TRK	Tyrosine kinase receptor
TRPC6	Transient receptor potential cation channel subfamily C member 6
VCR	Vincristine
w/v	Weight per volume
w/w	Weight per weight
WB	Western blot
WD40	Tryptophan-aspartic acid dipeptide repeats
wt	Wildtype
zCoro2ba	Zebrafish Coro2ba
ZFYVE28	Zinc Finger FYVE Domain-Containing Protein 28/LST2
ZO-1	Zonula occludens-1
α-SMA	Alpha smooth muscle actin

1 INTRODUCTION

This chapter provides background information to understand the following experimental chapters and studies investigating the role of new podocyte-associated proteins in the renal ultrafiltration barrier. Here the relevance of this thesis and the current knowledge in the field are addressed.

1.1 CHRONIC KIDNEY DISEASE – RELEVANCE IN HEALTH AND GLOBAL COST

According to the NIH's NIDDK (National Institute of Diabetes and Digestive and Kidney Disease), kidney disease kills annually more than 47 000 people in America annually, which is more than breast or prostate cancer does (NIH). Globally the prevalence of CKD is approximately 14%, with an estimated cost of over 1 trillion dollars per year (Hill et al. 2016, Stenvinkel 2010).The prevalence for CKD increases with age and is up to 35% for people above 70 years (O'Callaghan, Shine and Lasserson 2011). Risk of CKD is also associated with gender, as the prevalence for CKD is higher in women than in men. One factor contributing to the gender difference could be that women live longer and therefore represent a higher portion of individuals in the aging high risk group. The main causes of CKD are obesity, hypertension, diabetes mellitus and specific renal diseases. The high mortality and morbidity of CKD is mostly due to ensuing cardio vascular diseases (CVD), often leaving the patients when hospitalized with poor quality of life (Go et al. 2004, Hill et al. 2016). End stage renal disease (ESRD) is the final stage of CKD and only treatable with dialysis or transplantation and the prognosis for patients with ESRD is quite dire (Hill et al. 2016, Collins et al. 2009). Therefore, early detection of CKD is important, however the disease is unfortunately often asymptomatic and undetected in the absence of clinical routine screens. Possible screening methods include the determination of the estimated glomerular filtration rate (eGFR), testing for albuminuria, electrolyte or urine sediment disorders and histological imaging for structural abnormalities. The international KDIGO (Kidney Disease Improving Global Outcomes) organization has developed a staging system for kidney disorders, relying on GFR and albuminuria as the best index for kidney function in injury and health (Levey, Becker and Inker 2015). Table 1 shows the classification and definition of kidney disease by the KDIGO.

1.2 RENAL ULTRAFILTRATION

Kidneys are responsible for the filtration of the blood and generate up to 180 l of primary urine daily from blood entering the renal circulation. This means with an average blood plasma volume of 3 l is filtered up to 60 times per day. The functional structure accomplishing the filtration and subsequent urine modification is the nephron. Ultrafiltration occurs in the glomerular tuft which is surrounded by the Bowman's space and capsule. The capillary lumen of the glomerulus is lined by fenestrated endothelial cells. The glomerular basement membrane (GBM) surrounds the fenestrated endothelium and together with the terminally differentiated podocytes and their small intricate foot processes (FPs) as outer layer, comprise the glomerular filtration barrier (GFB). The third cell type of the glomerular tuft are mesangial cells, which are embedded in the mesangial matrix and provide structural integrity to the capillaries of the glomerular tuft. The contractile mesangial cells are able to biomechanically control the capillary tension through the GBM (Kurihara and Sakai 2017). The GBM is created by the attached podocytes and fenestrated endothelial cells, which produce and secrete the extracellular matrix proteins collagen IV, laminin and fibronectin.

Table 1: Definition and staging of kidney disease according to the KDIGO. Adapted from Levey et al. (Levey et al. 2015).

	Acute Kidney Injury	**Acute Kidney Disease and Disorders**	**Chronic Kidney Disease**
Definition	Within 2-7 days	≤3 Months	>3 Months
Functional criterion	Increase in serum creatinine by 50% within 7 days or increase in serum creatinine by 0.3 mg/dL (26.5 µmol/L) within 2 days or urine output <0.5 mL/kg/h for 6 hours	Glomerular filtration rate <60 mL/min/1.73 m2 or Decline in glomerular filtration rate by >35% times baseline or Increase in serum creatinine by >50% times baseline	Glomerular filtration rate <60 mL/min/1.73 m2
Structural damage criterion	None required	Marker of kidney damage (albuminuria, hematuria, or pyuria are most common)	Marker of kidney damage (albuminuria is most common)
Examples	Decreased kidney perfusion (prerenal disorders) Urinary tract obstruction (postrenal disorders) Intrinsic kidney diseases (acute tubular necrosis, acute interstitial nephritis)	Acute and rapidly progressive glomerulonephritis Acute presentations of nephrotic syndrome Acute pyelonephritis Partial obstruction of the urinary tract	Diabetic kidney disease Hypertensive nephrosclerosis Chronic glomerulonephritis Chronic interstitial nephritis Chronic pyelonephritis Polycystic kidney disease

The ultrafiltrate passes through the GFB into the Bowman's space and from there travels through a tubular system, comprised of the proximal tubule, the thin descending and thick ascending loop of Henley and the distal tubule into the urinary tract. The tubular tract facilitates the reabsorption of salts and molecules from the filtrate back into the bloodstream and also the secretion of waste metabolites such as urea and uric acid into the filtrate for excretion. Other than blood filtration and cleansing the body of toxins and waste metabolites, the kidney has many other important functions including blood pressure regulation and hormone production. Figure 1 shows the schematic structure of the kidney, the nephron and the glomerulus.

1.3 IN VIVO MODELS IN KIDNEY RESEARCH

A big challenge in kidney research is to find adequate research models, which satisfyingly mimic the function of human kidneys. The most commonly used animal models in many fields are rodents (mice and rats), including in kidney research, although many translational challenges exist (Rabe and Schaefer 2016). For instance, the standard strain used for generating knockout mouse lines, C57BL/6, is known to be very resistant to developing kidney damage, such as proteinuria or glomerulosclerosis, making it a difficult model to study relevant human-oriented disease mechanisms (Ma and Fogo 2003, Ishola et al. 2006). Nevertheless, mice are often the easiest accessible tool due to well established transgenic technologies and lack of alternatives.

An *in vivo* alternative to rodents is the zebrafish (*danio rerio*), which is often used to study developmental effects due to the possibilities for non-invasive monitoring. The fertilized embryos can be kept translucent by adding an inhibitor 1-Phenyl-2-thiourea (PTU) to the water and easily studied with a microscope (Karlsson, von Hofsten and Olsson 2001). Although the zebrafish is more distant

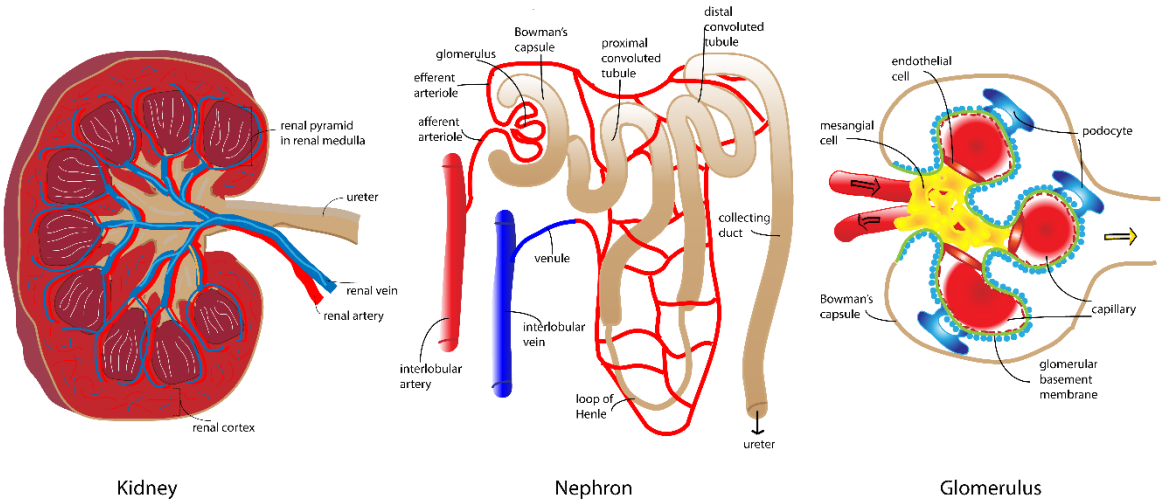

Figure 1: Illustration of a kidney cross section and the cortex located nephron. The cross section of the glomerulus shows the podocytes, mesangial cells and the fenestrated endothelia.

from the human in the phylogenetic tree of the vertebrate clade than rodents, it has been established that many aspects of renal physiology remained conserved. Knock-down of various monogenetic human nephropathy drivers in zebrafish larvae has been shown to lead to the development of disease patterns similar to the reflective human disease histology (Ebarasi et al. 2011, Kramer-Zucker et al. 2005). Thus, zebrafish provides an alternative for studying *in vivo* molecular mechanisms of renal physiology.

Kidney and glomerular tissue biology is very complex and therefore *in vivo* models are a necessity to dissect molecular mechanisms of normal biology and disease pathogenesis. *In vitro* alternatives, such as the use of immortalized podocyte cell lines is, however, very popular. Existing podocyte cell lines phenocopy poorly the molecular fingerprints and morphological patterns of *in vivo* podocytes and they can answer only a very limited number of questions. The relevance of these studies should always be carefully related to *in vivo* findings.

1.4 THE PODOCYTE ACTIN CYTOSKELETON IN HEALTH AND DISEASE

In over 70% of ESRD cases, aberrant function of renal glomeruli is the primary etiologic factor. In particular, it is the glomerular podocyte cells that seem to be of great importance. This is emphasized for instance by the fact that genetic mutations of podocyte specific proteins are causative for many hereditary diseases in man (Patrakka and Tryggvason 2009).

As terminally differentiated and highly specialized epithelial cells with unique arborized structure, podocytes have no proven capability of proliferation. Their functional contribution lies partly in their structural arborization. Podocytes have a cell body with protruding major processes (MP) and further protruding delicate FPs (Greka and Mundel 2012). The cytoskeleton of the FPs is formed by bundles of actin and possesses, unlike the cell body and MPs, no microtubular filaments (Moeller 2007, Ichimura, Kurihara and Sakai 2003). The FPs interdigitate and form filtration slits, which are bridged by slit diaphragms (SD). The slit diaphragm seems to be formed at least in part by overlapping type I transmembrane nephrin-NEPH1 (nephrin-like protein 1) molecules of the immunoglobulin (Ig-) superfamily. Intracellularly, nephrin and NEPH1 have tyrosine sites which are substrates for phosphorylation by the src-kinases fyn and yes, and also the small adaptor protein nck (Jones et al.

2006, Li et al. 2004, Simons et al. 2001). Other than the kinases binding with their SH2 domains, nephrin also interacts with podocin, Iq-motif containing GTPase-activating protein (IQGAP2), CD2-associated protein (CD2AP) and phosphoinositide 3-kinase (PI3K) with its SH2-domain and TRPC6, a Ca^{2+} cation channel binding both to nephrin and podocin at the SD (Patrakka and Tryggvason 2007, Reiser et al. 2005).

These proteins provide either scaffolding support to maintain the slit diaphragm and/or provide connection to the actin-cytoskeleton structurally or via signaling. CD2AP interacts with nephrin and podocin but also binds to F-actin capping protein (CAPZ) or cortactin, both of which orchestrate actin assembling (Faul et al. 2007, Schwarz et al. 2001). Additionally, CD2AP binds with its N-terminal SH3 domains to the C-terminus of Rac1, a member of the small Rho-GTPases family, which regulates cell adhesion and migration through the actin cytoskeleton (van Duijn et al. 2010, Mouawad, Tsui and Takano 2013).
Nck binds via its SH2 domain to the fyn activated phosphotyrosines of nephrin, which in turn binds to neuronal Wiskott–Aldrich syndrome protein (N-WASP) via its SH3 domain. N-WASP then recruits Arp2/3, a protein responsible for actin bundle branching (Jones et al. 2006, Blasutig et al. 2008, Verma et al. 2006). It is through this mechanism that nck provides an additional link between the slit diaphragm and the actin cytoskeleton.
IQGAP2 and PI3K also connect the SD to the actin cytoskeleton. The phospho-tyrosine dependent interaction of PI3K with nephrin results in the activation of Akt and cofilin, which are both actin cytoskeleton modulating proteins (Huber et al. 2003, Garg et al. 2010). IQGAP1 regulates the actin cytoskeleton through several ways. Firstly, IQGAPs are able to homodimerize and crosslink actin bundles via their calponin-homology domains (CHD). The oligomerization of IQGAP seems to be enhanced by the interaction with the small Rho-GTPases Rac1 and Cdc42, which are strong players in actin organization (Fukata et al. 1997). IQGAP can also bind Arp2/3 and affect the complex with N-WASP, WAVE and many other proteins involved in actin bundle branching (Watanabe, Wang and Kaibuchi 2015, Rotty, Wu and Bear 2013). It also has been shown that TRPC6 mediated Ca^{2+} influx impacts the actin dynamics of the podocyte FPs (Li et al. 2005, Moller et al. 2007).

The SD is of great importance for the maintenance of the glomerular filteration barrier. It is speculated that overlapping nephrin/NEPH1 molecules from adjacent foot processes form a sieve that contributes to the size-selective properties of the glomerular filter (Grahammer et al. 2016). Other than size selection, the anionic glycocalyx surrounding the podocytes and GECs is believed to contribute to the charge-selective properties of the barrier. One of the most important proteins of the glycocalyx is podocalyxin (Kerjaschki, Sharkey and Farquhar 1984, Pavenstadt 1998).
The SDs and the FPs provide a connection between adjacent podocytes, however alone they are not enough to withstand all of the mechanical forces occurring at the GFB. The fluid shear stress in the filtration slits, the Bowman's capsule and the circumferential wall stress between podocyte and GBM are considerable forces working against the podocyte, pushing it away from the GBM (Endlich and Endlich 2012). Naturally, there are more mechanisms involved keeping podocytes attached to the GBM and to each other. For example, the SD is supported by cadherin molecules like FAT1, which are anchored through the tight junction molecule zonula occludens-1 (ZO-1) to the actin network (Moeller et al. 2004, Yaoita et al. 2005).

The human GBM is around 300-350 nm thick and consists mainly of collagen IV, laminin and fibronectin, which is secreted by adjacent podocytes and the fenestrated endothelium (St John and Abrahamson 2001, Miner 2012). Major mechanical attachment to the GBM is formed by focal

adhesion (FAs) clusters of the integrin dimer α3β1 binding to laminin in the GBM (Bouaouina, Harburger and Calderwood 2012, Sachs and Sonnenberg 2013). Integrins are type I transmembrane proteins, which can be "switched on and off". Alone integrins do not possess a catalytic ability, however they are activated upon binding to extracellular matrix (ECM) proteins such as fibronectin (outside-in signaling) or through phosphorylation and dephosphorylation of the intrinsic NPXY-motif by proteins of the FA scaffold (inside-out signaling) such as tallin, kindlin or paxillin and filamin (Bass 2011). Integrin activation always goes hand-in-hand with the dimerization of an α- and β-unit, which then recruits surrounding integrin-dimers to form a cluster of FA. Some of the FA scaffolding proteins, like talin are able to directly bind actin and link the transmembrane integrin as an anchor between the cytoskeleton and the ECM (Xie et al. 2010, Barczyk, Carracedo and Gullberg 2010). Figure 2 shows schematically the interactions of various proteins in the SD and podocyte FP with the GBM.

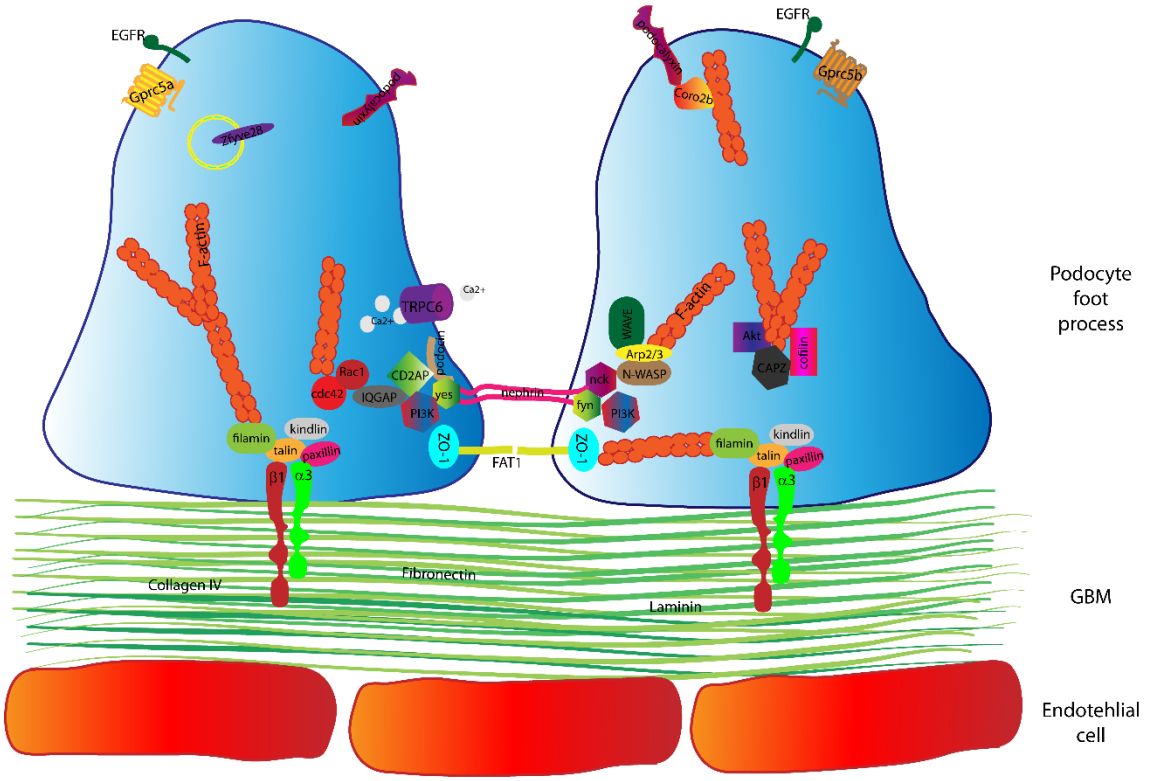

Figure 2: Podocyte foot processes and their proteins are involved in the interaction with the GBM and the SD.

1.5 PROTEINS INVOLVED IN ACTIN REGULATION CAUSE PODOCYTE INJURY

Many functions of the podocyte are closely related to the regulation of the actin cytoskeleton. The SD is directly linked to the actin cytoskeleton and the podocyte attaches to the GBM to brace against physical forces through integrin-mediated FAs. It is not surprising that many diseases have been found to be caused by mutations or aberrant functions of proteins involved in the regulation and pathway machineries of the cytoskeleton. One of the first visible signs of the onset of kidney disease is the effacement of podocyte FP, which are maintained by the actin cytoskeleton (Murphy, Moretta and Jukkola 1979). When the podocyte loses the connection to the GBM, it detaches and is flushed away with the urine. As they are terminally differentiated, the neighboring podocytes cannot proliferate. Instead, they usually compensate by growing in size and develop hypertrophy. However, over time

this compensatory mechanism becomes during time maladaptive and ultimately leads to leakage of proteins such as albumin through the filtration barrier. This is clinically detectable as proteinuria or albuminuria (Kriz and LeHir 2005, Kriz et al. 2014). One disease affecting the human glomerulus due to a genetic mutation is Alport syndrome. In patients with Alport syndrome the collagen IV gene is mutated, which ultimately leads to progressive renal failure, hearing loss and ocular abnormalities of the retina. Due to the defective collagen IV, the GBM is impaired and leads to ruptures of the glomerular capillaries and subsequent hematuria, an initial manifestation of the syndrome (Kashtan 1999, Liapis, Foster and Miner 2002, Kashtan 2017).
In hereditary human focal segmental glomerulosclerosis (FSGS), mutations of the Ca^{2+}-ion channel TRPC6 lead to renal failure. It is speculated that the increased Ca^{2+} -influx leads to reorganization of the actin cytoskeleton in podocyte FPs and subsequent FP effacement. Another protein shown to be causative for FSGS when deleted specifically in the podocytes of mice is integrin linked kinase (ILK) (El-Aouni et al. 2006). ILK induces Wnt-signaling and the phosphorylation of α-actinin, altering the actin arrangement and podocyte attachment (Blattner and Kretzler 2005, Teixeira Vde et al. 2005).
Ablation of integrin α3 or β1 in mice is developmentally lethal. *Itgb1* (integrin β1) null mice embryos are aborted after the blastocyst stage during the embedding and invasion into the uterine basement membrane (Fassler and Meyer 1995). Deletion of *Itga3* (integrin α3) causes the pups to die shortly after birth due to renal and respiratory failure. The mutants showed general defects of vascular branching in the bronchi and the glomerular capillary loops. Lack of α3 resulted in a disorganized GBM and the FPs were not formed, affecting the glomerular development (Kreidberg et al. 1996).
Three identified human patients with a homozygous α3 mutation showed disorganized basement membranes and faulty barrier functions in the kidney, the lung and skin. The renal failure resembled the symptoms of congenital nephrotic syndrome and the patients died due to the lung disorder (Has et al. 2012).
Recently, it has been shown that the beneficial effects of chemotherapeutic agent vincristine (VCR) in frequent relapse nephrotic syndrome and steroid-dependent nephrotic syndrome is due to its downregulation of pathologically elevated α3β1 integrin. Yin *et al.* showed through quantitative PCR analysis that the expression levels of α3β1 integrin and FAK are elevated in the Adriamycin (ADR)-nephropathy rat model. Those levels decrease when low dosages of VCR are administered *in vivo* and *in vitro* and the disrupted actin cytoskeleton phenotype is rescued (Yin et al. 2017).

Two other examples of agents targeting pathways of the actin cytoskeleton to rescue from renal damage are Bis-T-23 and fasudil. Bis-T-23 is a promoter of actin-dependent dynamin oligomerization and has been shown to improve or attenuate disease progression in mice and zebrafish models of transient kidney disease and CKD. The increased dynamin oligomerization initiated by Bis-T-23 results in increased actin polymerization, stress fiber and FA formation. Thus supporting the actin-dependent structure of the FP and the renal health, even when RhoA is downregulated in cultured podocytes. This suggests that dynamin may be able to drive FA maturation independent of the RhoA pathway (Schiffer et al. 2015, Gu et al. 2017).
Fasudil is an inhibitor of ROCK, a rho-associated protein kinase upstream of several actin regulating pathways which has been connected to the progression of renal fibrosis. Fasudil slowed disease progression, decreasing glomerusclerosis and interstitial fibrosis in streptozotocin (STZ)-induced diabetic rats (Nagatoya et al. 2002, Park et al. 2011, Komers et al. 2011).

It becomes apparent that the podocyte and the intricate actin cytoskeleton within the FPs is of great importance to renal health and function. Direct targeting of actin regulating pathways in various

disease models seems to confer reno- and podocyte-protective value, offering an attractive possibility for patient treatment. The actin cytoskeleton is a structural and regulatory component of all mammalian cells and therefore may seem like a less suitable drug target, however the highly specialized state of podocytes comes with a specialized pattern of protein expression. Finding a podocyte-specific or enriched regulator of the actin cytoskeleton, which can in turn be targeted by inhibition or activation, could help to arrest or counteract disease progression in CKD. Figure 3 illustrates the podocyte FPs in health and disease.

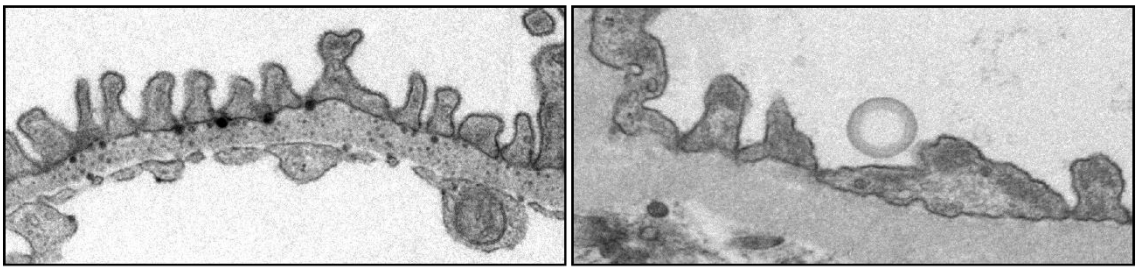

Figure 3: TEM images of normal podocyte FPs (left) and effaced podocyte FP with thickened GBM (right). Both images have the same magnification.

1.6 ROLE OF TGF-β IN NEPHROPATHIES

In many nephropathies, resulting from wide ranging and often still undiscovered causative effects, it is the glomerulus and their podocytes that are the first to show injury and malfunction. But irrespective of the functional compartment from which the disease originates from, when patients progress to CKD the histological changes converge. Renal diseases like DN, FSGS, IgA nephropathy or lupus nephritis all show accumulation of ECM and tubulointerstitial injury leading to interstitial fibrosis (Bohle et al. 1989, Nath 1992). Almost invariably connected to fibrosis is the elevated expression of transforming growth factor β (TGF-β). In most nephropathies associated with increased matrix accumulation, the enhanced expression of all three TGF-β isoforms or the upregulation of their receptors TGF-β type I & II receptor (TGFβRI & TGFβRII) is common (Yamamoto et al. 1998, Sharma et al. 1997, Yamamoto et al. 1996).

TGF-β is secreted in an inactive form that is activated through different mechanisms, including proteolytic cleavage into two homodimers (ten Dijke and Arthur 2007). In its active form TGF-β interacts with its receptors in a cellular composition and signal specific manner. Classically, the signal transduction is either canonical (SMAD-dependent) or non-canonical (SMAD independent) signaling (Zhang 2017). Upon ligand binding to TGFβRII, the constitutively active receptor dimerizes with and serine/threonine phosphorylates TGFβRI, which in turn phosphorylates and activates one of the R-SMADs (receptor regulated SMADs: SMAD2/3 & SMAD1/5/8). The activated R-SMAD complex then forms a larger complex with the common co-transcription factor SMAD4 and shuttles into the nucleus to initiate the corresponding gene transcription. The pathway is feedback regulated by I-SMADs (inhibitory SMADs: SMAD6/7), which either compete for binding to TGFβRI with the R-SMADs or facilitate the ubiquitination and ensuing degradation of SMAD-complexes (Zhang 2018, Gotovac et al. 2018, Massague 2012).

Depending on the receptor combination and in what tissue it binds, TGF-β alters the cellular protein expression through SMAD-dependent or independent signaling, modulating cellular processes such as proliferation, motility, extracellular matrix assembly, embryonic development, regulation of the immune system and inflammation (Mishra et al. 2005, Roberts et al. 2006). When properly regulated

in homeostasis TGF-β is a beneficial and necessary growth factor for mammalian organisms, but when dysregulated leads to a magnitude of functional imbalances. Alone, the administration of high doses of human TGF-β or the targeted overexpression of TGF-β in rat kidneys results in glomerulosclerosis and accumulation of ECM (Terrell et al. 1993, Isaka et al. 1993).

The TGF-β pathway has been targeted from various angles as means of therapeutic intervention. An example is the small molecule pirfenidone, which was tested in human clinical trials (Cho et al. 2007, Sharma et al. 2011). Initially, pirfenidone was shown to reverse around 50% of tubulointerstitial fibrosis and reduce TGF-β1 expression by up to 80% in salt-depleted and cyclosporine A (CsA) administered rats (Shihab et al. 2002). In human trials it failed to reproduce the same reno-protective effects as have been seen in rats and hamsters (Iyer, Gurujeyalakshmi and Giri 1999). In a group of FSGS and DN patients , administration of pirfenidone did not affect proteinuria or blood pressure but attenuated the monthly change of the eGFR rate (Sharma et al. 2011, Cho et al. 2007, Isaka 2018). Other small molecules such as decorin or fresolimumab, target different aspects of the TGF-β signaling, also had a similarly poor success in ameliorating renal fibrosis (Isaka 2018).
Taken together, although many approaches have not yielded a positive outcome for anti-TGF-β therapy in CKD, it still remains an attractive potential intervention and further research in this field is warranted.

1.7 EGF RECEPTOR PATHWAY

CKD progression and loss of kidney function is histologically mainly measured by the extent of ECM expansion, inflammatory cell accumultion and increasing fibrosis. Another molecule associated with most of these processes is the epidermal growth factor receptor (EGFR also known as ERBB1). EGFR signaling governs pathways leading to cell proliferation, ECM regulation and inflammatory response. In the kidney EGFR is expressed both in the glomerulus and tubulointerstitium (Yoshioka et al. 1990). In disease models, the modulation of EGFR signaling has been reported to have both positive and detrimental effects. While the suppression of EGFR is mostly associated with beneficial effects in progressive nephropathies through the amelioration of fibrosis (Rayego-Mateos et al. 2018), whereas in acute kidney injury (AKI) elevated levels of EGFR promoted recovery of renal tubular cells through enhanced proliferation and protection from apoptosis (Chevalier, Goyal and Thornhill 1999, Zhuang et al. 2007).
These findings confirm that EGFR is a multifactorial player. There are already 7 recognized EGFR ligands, including EGF, transforming growth factor α (TGFA) and heparin-binding EGF-like growth factor (HBEGF) and newly added to the list is a connective tissue growth factor (CTGF/CCN2) amongst the soluble ligands triggering direct activation (Rayego-Mateos et al. 2018). Activation of EGFR leads to a conformational change allowing it to homo- or heterodimerize with one of its family members ERBB2, ERBB3 or ERBB4, and autophosphorylate its intracellular tyrosine residues (Olayioye et al. 2000). The phosphorylated tyrosine residues are recognized by signaling molecules with SRC- homology domains and can trigger different signaling pathways, such as RAS/RAF, MEK/ERK and MAPK, or activate transcription factors such as NF-κB or c-JUN (Holbro and Hynes 2004, Yu et al. 2002, Rojas, Yao and Lin 1996).
Besides having a multitude of ligands and co-receptors for direct activation, EGFR can also be transactivated by other membrane bound proteins such as GPCRs, integrins, cytokines and other tyrosine kinase receptors (TKRs) (Moghal and Sternberg 1999, Hackel et al. 1999). In particular, the crosstalk between EGFR and GPCRs has been associated with the regulation of inflammatory

processes (El Zein, D'Hondt and Sariban 2010).
Clinical trials targeting the EGFR pathway have been conducted, although mainly in cancer therapy due to elevated EGFR expression being found in 90% of tumors (Martinez-Useros and Garcia-Foncillas 2015). One monoclonal antibody developed against the extracellular kinase domain of EGFR, cetuximab, showed promise by improving survival rates of lung and colorectal cancer patients when used in combination with chemotherapy (Pirker et al. 2009, Jonker et al. 2007). In the context of renopathies, studies have been performed in rodent models. In mice, blocking of EGFR kinase decreased the formation of cysts and improved renal function in an autosomal recessive polycystic kidney model (Richards et al. 1998). In diabetic rats, the blockade of EGFR increased glomerular size and lowered the proliferation of tubular epithelial cells (Wassef, Kelly and Gilbert 2004).
Taken together, although clinical studies proving that EGFR targeting can be beneficial for nephropathies have not yet been performed, it offers a wide range of molecular targets for intervention. The tissue-specific expression of EGFR, multiple co-receptors and direct ligands can provide a possibility for tissue or cell-specific regulators of the EGFR pathways, which can in turn can be used as a target in CKD in a cell/tissue specific manner.

1.8 NF-κB PATHWAY

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a DNA binding protein complex known to activate genes regulating cellular mechanisms including proliferation, immune responses, apoptosis and organogenesis. Stimuli triggering the activation of the transcription factor NF-κB include cytokines such as tumor necrosis factor alpha (TNFα) or interleukin 1-beta (IL-1β), bacterial lipopolysaccharides (LPS) stimulation of toll-like receptors (TLRs), and cellular stressors such as DNA damage due to ultraviolet irradiation or reactive oxygen species (ROS) (Schmitz et al. 2004, Fitzgerald et al. 2007, Basu et al. 1998, Silverman and Maniatis 2001). The signaling cascades associated with the the NF-κB transcription factor family, as well as its function in immune responses, have been evolutionary conserved throughout the mammalian clade (Ghosh and Karin 2002).
Five different NF-κB proteins exist (RelA/p65, RelB, c-Rel, p100/p52 and p105/p50), defined by a N-terminal DNA-binding domain (DBD) and three (the Rel's) also possess also one or two C-terminal transactivation domains (TADs) for targeted gene transcription. The DBD domain also facilitates dimerization and contains the nuclear localization sequence (NLS) to which also 8 mammalian inhibitory IκB proteins with their inherent ankyrin repeats can bind (Schmitz and Baeuerle 1991, Saccani, Pantano and Natoli 2003, Baeuerle and Baltimore 1996, Huxford, Malek and Ghosh 1999, Malek et al. 2001).
Canonical activation of the NF-κB pathway begins with an extracellular signal such as TNFα binding to the TNFR receptor in turn activating the IκB kinase complex (IKK), which is comprised of a heterodimer of IKKα and IKKβ plus a regulatory subunit called NEMO/IKKγ (Ghosh and Karin 2002). IKK phosphorylates IκBα at serines 32 and 36, which then in turn is recognized by a SCF ubiquitin ligase. After polyubiquitinylation IκBα is subject to proteasomal degradation (Yaron et al. 1998) and the freed NF-κB then shuttles into the nucleus binding to co-factors and the DNA and initiating transcription. NF-κB also activates expression of IκBα, its own repressor, and is thereby regulated by an auto feedback loop (Nelson et al. 2004).
Other than the variuos possible dimerization options and different activating stimuli, NF-kB is also subject to phosphorylation and acetylation/deacetylation to determine its DNA-binding properties and interaction with co-transcription factors. P65/RelA has six serines and two tyrosines, which can be phosphorylated or dephosphorylated by various kinases and phosphatases. The phosphorylation status

of serine 276 on p65 determines whether histone deacetylase-1 (HDAC-1) or the acetylase CBP/p300 interacts with p65 to cause either shut down or co-activation, respectively (Schmitz et al. 2004, Zhong, Voll and Ghosh 1998, Zhong et al. 2002).
The NF-κB pathway, like the TGF or EGF pathway, has multiple points of regulation and its aberrant activation is involved in numerous diseases. Therefore, manipulation of the pathway could have beneficial effects in many disease processes. However, the targeting of such a universal pathway poses significant risks for off-target effects. Instead, discovery of cell specific regulation mechanisms of the NF-κB pathway could reveal better options for therapeutic intervention.

1.9 CORONIN CORO2B

More than 723 different coronins have been identified in eukaryotes, and seven are found in mammals (Eckert, Hammesfahr and Kollmar 2011). The seven mammalian coronins can be divided into 3 subclasses based on sequence homology, however all of them have the common conserved structure of a (de Hostos 2008, Xavier et al. 2008) N-terminal extension followed by a seven-bladed β-propeller, made of five canonical and two noncanonical WD40 repeats. The two noncanonical WD40 repeats interact with actin and the plasma membrane. After the β-propeller is the C-terminal extension with a unique region of various length, however, in all but coronin7, a coiled coil domain is common, which can directly interact with actin and the Arp2/3 complex to undergo homo-oligomerization (Liu et al. 2016b, Rybakin and Clemen 2005, McArdle and Hofmann 2008).
Woroniecka *et.al.* performed a transcriptome analysis of healthy and DN glomeruli and tubulointerstitium and detected Coro2b (Coronin 5 or Clipin C) as one of the most highly enriched proteins in the glomerular fraction compared to the tubulointerstitium. Additionally, the analysis revealed that Coro2b is one of the most downregulated proteins in DN glomeruli compared to healthy glomeruli (Woroniecka et al. 2011).
Coro2b, along with coronin 4 (Coro2a; IR10; Clipin B; WDR2) and coronin 6, belongs to the type II class of mammalian coronins (Liu et al. 2016b). Coro2a and Coro2b both bind to F-actin stress fibers and localize within FAs. Expressed most highly in the brain, Coro2b was shown to bind to vinculin, a major component of FAs, and localize to neurite tips (Nakamura et al. 1999, Huang et al. 2011). A recent study in kidney tissue revealed Coro2b is also expressed in human and mouse podocytes (Rogg et al. 2017). Coro2b was suggested to be involved in the recruitment of CFL1 (cofilin-1), an actin fiber depolymerizing protein, to FAs. Additionally, constitutive Coro2b KO mice showed partial protection in a FSGS model induced by doxorubicin administration (Rogg et al. 2017).
Recently, mutation of the Coro2b gene was found via whole genome sequencing of a patient with a ciliopathy-like phenotype, and was proposed to be a novel candidate in the regulation of ciliopathy related diseases(Castro-Sanchez et al. 2017). Coro2a was shown to co-localize to the nuclear receptor corepressor (NCoR) complex and be involved in an actin-dependent mechanism for the de-repression of inflammatory response genes (Huang et al. 2011). On the other hand, the third member of the type II class coronins, coronin 6, was identified in neuromuscular junctions (NMJ), which are essential for neuromuscular transmission and muscle activity (Chen et al. 2014, Dobbins et al. 2006).

1.10 GPCRS

G-protein coupled receptors (GPCRs) are 7-α-helical transmembrane receptors and constitute, with over 800 members, the biggest family of membrane surface proteins in vertebrates. Intracellularly, they are connected to a heterotrimeric αβγ guanine nucleotide binding protein (G-protein), which is activated upon conformational change of the GPCR. Conformational change is induced upon agonist

or ligand binding to the extracellular N-terminal domain and the trimeric G-protein exchanges guanine diphosphate (GDP) to guanine triphosphate (GTP), releasing the GTP binding α-unit to initiate downstream signaling cascades. Extracellular signals including hormones, neurotransmitters, peptides, and odorants regulate multiple physiological processes via GPCR binding, such as cell growth and differentiation, metabolism and many others (Heldin et al. 2016, Karnik et al. 2003, Dunham and Hall 2009). From a pharmacological point of view, GPCRs are attractive drug targets and constitute almost 30% of the market share of currently used drugs in the clinic (Nieto Gutierrez and McDonald 2017). Recently, β-arrestin, a negative regulator of GPCRs through internalization and desensitization, was implied to play a role in podocyte autophagy in DN injury (Liu et al. 2016a). They showed that β-arrestin 1 and 2 were elevated in STZ-induced diabetic mice and that deficiency of either of the β-arrestins would ameliorated the renal injury (Spiegel 2003, Liu et al. 2016a). Finding a podocyte or a glomerulus-specific GPCR involved in renal injury could offer an attractive target for the treatment of CKD.

1.10.1 Gprc5a

The G-protein coupled receptor class C group 5 member A (Gprc5a), also known as retinoic acid-induced gene 3 (RAI3), is one of four members (Gprc5a, Gprc5b, Gprc5c, Gprc5d) of a group of orphan GPCRs lacking known small molecule ligands. As the name implies, the expression of the four Gprc5's is induced by retinoic acid, a metabolite of vitamin A_1 (Duester 2008, Kurtenbach et al. 2011, Robbins et al. 2000).

Gprc5a expression is not exclusively induced by retinoic acid, but is also regulated by other factors, including BRAC1, FOS, JUN, cAMP and the tumor suppressor p53 (Zhou and Rigoutsos 2014). Additionally, Gprc5a can be subject to N-glycosylation, phosphorylation, ubiquitinylation and is a target of microRNAs (miRNAs) (Wagner et al. 2011, Danielsen et al. 2011). For example miR-342 suppresses Gprc5a expression in glioma and suppresses the gliomal cell proliferation (Wang et al. 2019).

Gprc5a has been mostly studied in various types of cancer. However, the role of Gprc5a in cancer seems to be contradictory, as Gprc5a expression has been shown to be both beneficial or detrimental depending on which cell type the tumor originates. In gastric cancer, Gprc5a expression levels are highly elevated compared to the usually healthy mucosal tissue (Cheng et al. 2012). In breast cancer, Gprc5a acts as a tumor suppressor by inhibiting EGFR and its downstream pathway. Similarly, in non-small cell lung cancer (NSCLC) its upregulation is linked to the upregulation of p53, decreasing tumor viability and increasing apoptosis (Yang, Ma and Zhang 2016, Jin et al. 2017).

Gprc5a has also been implicated in the regulation of inflammation. In a study with Gprc5a KO mice, LPS administration resulted in higher levels of cytokines such as TNFα and IL-1β in the lungs of KO mice compared to the wt. Pulmonary inflammation was linked to dysregulated NF-κB signaling in the bronchioalveolar epithelium, as administration of NF-κB inhibitor IκBα ameliorated the inflammation in the Gprc5a KO lungs (Liao et al. 2015).

1.10.2 Gprc5b

Another member of G-protein coupled receptor class C group 5 receptor is Gprc5b Also known as retinoic acid-induced protein 2 (RAIG-2), it is mainly expressed in the central nervous system (CNS), the kidney, the pancreas and the testes (Robbins et al. 2002, Brauner-Osborne and Krogsgaard-Larsen 2000). In the pancreas, Gprc5b was shown to be a negative modulator of insulin secretion. Tyrosine phosphorylated Gprc5b has been shown to recruit Fyn via its SH2 domain, which in turn

phosphorylates sphingomyelin synthase 2 (SMS2). Phosphorylated SMS2 is prone to ubiquitinylation and results in increased diacylglycerol (DAG) production, inhibiting insulin secretion through JNK activation (Kim, Greimel and Hirabayashi 2018, Soni et al. 2013, Kim et al. 2012).
Gprc5b has also been connected to NF-kB signaling in adipose tissue. Phosphorylated Gprc5b recruits Fyn, which activates NF-κB. Caveolin-1 (Cav-1) blocks Gprc5b phosphorylation by directly binding to its cytoplasmic tail to the C-terminal domain of Cav-1 (Kim and Hirabayashi 2018). Additionally, Gprc5b deficient mice are protected from diet-induced diabetes and insulin resistance due to less inflammation in white adipose tissue (Kim et al. 2012).
Two other studies with Gprc5b KO mice reported behavioral abnormalities and deffective long term motor learning due to disrupted synaptic plasticity. Gprc5b deficient Purkinje cells developed axonal swelling in deep cerebellar nuclei, which could be ameliorated by pharmaceutical reduction of ROS (Sano et al. 2018, Sano et al. 2011).

1.11 ZFYVE28

Zinc finger FYVE domain-containing protein 28 (ZFYVE28), also known as human lateral signaling target protein homolog 2 (hLst2) is known to be a negative regulator of EGFR signaling. Via its FYVE domain, it is able to bind phosphatidylinositol 3-phosphate (PI3P) on endosomes and influences whether enclosed EGFRs will be degraded or recycled. Monoubiquitinylated ZFYVE28 inhibits the binding of PI3P on endosomes, which favors the recycling of the EGF receptor. Non-ubiquitinylated ZFYVE28 promotes the degradation of EGFR into the maturing endosomes (Yoo, Bais and Greenwald 2004, Mosesson et al. 2009).

1.12 CONCLUSION

CKD is a major global health challenge. Despite major advancements in differing medical fields, no curative strategies have been developed to deal with the increasing numbers of ESRD patients. The current available approaches rely on untargeted medication, dialysis and, in fortunate cases, transplantation. It is widely accepted that the glomerulus and the podocyte play a major role in CKD development and progression. In particular, it is the intricate actin cytoskeleton structure of podocyte FPs tha is one of the key structures affected in disease. The highly specialized structure and function of the podocyte comes along with a unique molecular machinery, which can potentially provide us with podocyte-specific regulators. Successful targeting of these regulators could allow us to develop cell-specific therapeutic options.

2 AIMS

The overall aim of this thesis was to investigate the role of new podocyte-associated proteins in the renal ultrafiltration barrier, with the ultimate goal of identifying possible new drug targets to treat glomerular disease.

The specific aims were:

Study I To characterize the role of Coro2b in the glomerulus and podocytes in health and disease.

Study II To study the role of Coro2b in glomerular development of the zebrafish.

Study III-IV To identify novel glomerulus-enriched GPCRs and to study their role in glomerular biology and diseases.

Study V To characterize the role of glomerulus-enriched protein ZFYVE28 in glomerular biology and diseases.

3 METHODOLOGICAL APPROACHES

This chapter briefly describes the main methods and materials used within this thesis. More detailed descriptions are found in the Materials and Methods sections of the respective publications and studies.

3.1 ETHICAL CONSIDERATIONS

3.1.1 Human samples

Kidneys were collected from patients who underwent nephrectomy due to renal tumors, and renal biopsies were collected from patients with DN, IgAN or MN collected at the Karolinska University Hospital (Stockholm, Sweden), in accordance with the approval of the regional Ethical Committee (Regionala Etikprövningsämnden i Stockholm 2010/579-31; 2017/58-31/4). Human fetal kidney samples used for immunofluorescence labeling were from 20-week old fetuses, aborted due to neural tube defects and hydrocephalus, and collected by the University Hospital Helsinki, Finland with approval from the local ethics committee of the University Hospital Helsinki, Helsinki, Finland (Dnro HUS 356/E7/2005, Dnro 80/E7/2002).

3.1.2 Mice

All animal-related methods were performed in accordance with the relevant guidelines and regulations, and were approved by the ethical Committee on Research Animal Care (Linköpings djurförsöksestiska nämd DNR41-15).

3.1.3 Zebrafish

Zebrafish were housed in accordance with the Swedish and European animal husbandry and ethical guidelines. All fish-related methods were performed in accordance with relevant guidelines and regulations and approved ethical permits (Stockholms Norra djurförsöksestiska nämd DNRN15-15).

3.2 CELL CULTURE

Human podocytes were cultured as previously described (Saleem et al. 2002). Overexpressing cell lines were generated by vector transfection with Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol and following antibiotic selection.

3.3 PCR, QPCR AND WB

DNA from mouse ear biopsies or fishtail biopsies was extracted in sodium hydroxide solution at 98˚C and subsequently neutralized in Tris-buffer. RNA was extracted from tissue samples preserved in Trizol (Invitrogen/ ThermoFisher) using the RNAeasy kit (Qiagen), according to the manufacturer's protocol. cDNA was generated using the iScriptase kit (Biorad). PCRs and qPCRs were performed according to standard protocols, with optimized adjustments of annealing temperature for the individual primers and elongation times dependent on amplicon size. For WB, tissue or cells were lysed with RIPA-buffer and processed according to established standard protocols. Up to 30µg of protein were loaded per gel pocket.

3.4 GLOMERULAR ISOLATION

Human and mouse glomeruli were isolated from kidney tissues through a combination of tissue mincing and washing through sieves of varying size, to size-select the glomeruli (Takemoto et al. 2002). Zebrafish pronephri were microdisected from 96h old podocin-GFP positive larvae.

3.5 IMMUNOFLUORESCENCE, ELECTRON AND SUPERRESOLUTION MICROSCOPY

Tissue samples were embedded in OCT, snap frozen in liquid nitrogen and stored at -80˚C before cryosectioning. For paraffin sections, fresh or fixed (24h incubated in 4% PFA (w/v) in PBS) tissue was paraffin embedded according to standard protocols and sectioned using a microtome. Slides of sectioned tissue were fixed with acetone at -20˚C and blocked with 5% goat serum (v/v) in PBS before antibody incubation (diluted in blocking solution). Immunoperoxide-labeled antibodies were visualized using a DAB Peroxidase (HRP) Substrate Kit (with Nickel), 3,3'-diaminobenzidine (Vector Biolabs #SK-4100) according to manufacturer's protocol. Images were acquired using confocal or wide-field light microscopes.
STED microscopy and optical clearing was carried out according to a previously published protocol (Unnersjo-Jess et al. 2016), with the some modifications: Kidneys were pre-fixed in 4% PFA in PBS for 24h at 4°C, then transferred to monomer solution (4% acrylamide, 0.25% VA-044 thermal initiator in PBS) at 4°C overnight, polymerized for 3h at 37 °C and cleared in clearing solution for 48h before immunostaining, mounting and imaging. Electron microscopy was performed using standard protocols, including fixation with 2.5% glutaraldehyde by the EM facility of the Karolinska University Hospital.

3.6 MESANGIAL INDEX AND SIGNAL INTENSITY SCORING

Semi-quantitative scoring of the signal intensity of antibody labelling in patient biopsies and PAS or HE stained histological mouse sections, were performed visually on a light microscope. The severity of sclerotic mesangial expansion or signal intensity were graded from none to strong on a scale of 0-3 on randomized picked glomeruli. Depending on the experimental purpose, either the sum or mean was calculated.

3.7 STZ INDUCED DIABETES MODEL

Diabetes was induced in 8-week old mice via intraperitoneal injections of streptozotocin (STZ, 50 mg/kg, Sigma-Aldrich) after 4 h of fasting for 5 consecutive days. Hyperglycemia was checked 12 days after the last injection and at the end of the experiment by measuring non-fasting blood glucose. Mice were checked weekly for their wellbeing and urine was collected every second week starting from 5 weeks after the last injection. 6µl per urine sample were loaded on SDS-gels to check for protein content. Mice were euthanized after 24 weeks.

3.8 PS INURY MODEL

Perfusion of 4-month old mice with protamine sulfate and heparin sulfate was performed as described previously (Garg et al. 2010). Perfused kidneys were fixed accordingly for either TEM or paraffin embedding.

3.9 MORPHOLINO KNOCKDOWN

Morpholinos were injected using standard procedures into podocin-GFP transgenic fish embryos (Ebarasi et al. 2009) or AB wt fish embryos. The morpholinos were designed by Gene-Tools, and administered to freshly fertilized zygotes up until the 2-cell stage of development. No more than 2 nl injection solution with a concentration of 100 mM morpholino were administered per embryo. Zebrafish embryos were kept in E3 medium with or without pigmentation inhibitor 0.003% w/v 1-phenyl-2-thiourea (PTU;Sigma-Aldrich) at 28.5˚C depending on the experiment.

3.10 PRONEPHRIC FILTRATION ASSAY

72h old larvae were received injections of 10-kDa rhodamine-labeled dextran and 500-kDa FITC-labeled dextran in 0.2 M KCl into the common cardinal vein, as previously described (Drummond et al. 1998). Post-injection, the larvae were left for 16h in E3 medium with PTU at 28.5 ˚C before being sacrificed, fixed in 4% PFA solution and further processed for JB4 plastic embedding.

3.11 JB4 PLASTIC EMBEDDING

Larvae were embedded and sectioned in JB4 resin according to previous published protocols (Sullivan-Brown, Bisher and Burdine 2011). In brief, after PFA fixation, the larvae were dehydrated through sequential increasing ethanol concentrations in PBS until 100% ethanol. Larvae were then kept overnight in JB-4 infiltration solution, rinsed with fresh JB-4 infiltration solution, placed into casting molds and left to polymerize into plastic blocks for further sectioning and analysis.

3.12 IN SITU HYBRIDIZATION

In situ hybridization was performed according to previously published protocols and visualized using standard light microscopy (Thisse et al. 2004).

4 RESULTS AND DISCUSSION

This chapter provides an overview of the conducted studies in this thesis. It discusses the main findings and the relevance of each study. The figures or tables mentioned are found in the respective manuscripts/publications attached to the thesis.

4.1 CORO2B IN HUMAN DN AND ITS INVOLVEMENT IN PROTAMINE SULPHATE-INDUCED FOOT PROCESS EFFACEMENT (STUDY I)

DN is a major health burden worldwide and its incidence is increasing along with the global diabetic epidemic. To date there is still no effective cure other than the transplantation of a donor kidney, however even then life expectancy is reduced. Amongst the major signs of injury during the development of DN is the damage and loss of podocytes. Podocytes, with their interdigitating FPs, are a critical part of the filtration barrier separating the blood and urine. The FPs are cellular protrusions upheld by the actin cytoskeleton, and its modulation has been shown to elicit renoprotective effects in disease models (Schiffer et al. 2015). Therefore, we decided to study the role of Coro2b, a podocyte enriched protein belonging to an actin cytoskeleton regulating protein family, in disease physiology.

Employing confocal microscopy and super resolution stimulated emission depletion (STED) microscopy we showed that Coro2b is a glomerulus and podocyte-enriched protein within the human and murine kidney cortex. Co-staining with the glomerular cell markers nephrin and podocalyxin in podocytes, CD31 in endothelial cells and platelet-derived growth factor beta (PDGFRβ) in mesangial cells, confined the expression of Coro2b to the podocyte (figure 1). Using STED microscopy in combination with a clearing method developed by our collaborator, we could show that Coro2b localizes centrally and toward the apical plasma membrane within the FPs and not basally in man and mouse (figure 2). Woroniecka *et al.* reported Coro2b as among the top 5 downregulated proteins in glomeruli of DN patients in comparison to control glomeruli (Woroniecka et al. 2011). Therefore, we analyzed the staining intensity of Coro2b in patient biopsies of DN, IgAN and MN to gain greater insights into the expression level of Coro2b in common nephropathies. Staining the same patient biopsies for synaptopodin as an indicator of podocyte loss, we could show that Coro2b downregulation occurred solely in DN and not in IgAN or MN, independent of the number of podocytes remnant in the glomeruli (figure 3). To explore the physiological role of Coro2b *in vivo*, we generated a constitutive and podocyte-specific KO mouse strains through Cre-lox recombination on B6J background. Both KO strains exhibited no obvious phenotype and showed a normal Mendelian birth distribution (figure 4). The induction of diabetes through STZ injections caused similar nephropathy in both podocyte-specific KO and wt control mice. However, in the PS injury model, which targets the podocyte actin cytoskeleton, significant differences between KO and control animals were observed. TEM showed that the KO animals lacking Coro2b in podocytes were partially protected from the development of FP effacement after PS perfusion and even recovery of normal FP structure through subsequent HS perfusion was compromised (figure 5&6).

The kidney is a complex organ containing different functional compartments, each consisting of a specialized subset of cells with unique properties and purpose. The podocyte is among the most unique cell types of the kidney, with its one-of-a-kind arborized morphology, demanding localization between blood and primary urine, and terminal differentiation rendering it incapable of further proliferation - somewhat reminiscent of neuronal cells. This high grade of specialization comes along with a tailored set of protein expression that offers potential targets for specific therapeutic modulation

without affecting the whole somatic system. Coro2b is almost ubiquitously expressed in low levels, except for the cerebral cortex and hippocampus, where it is highly expressed (www.proteinatlas.org). Coro2b has previously been shown to localize to FAs of neurite tips as well as in immortalized human podocytes, where it is also described as localizing to the ventral F-actin fibers (Rogg et al. 2017, Nakamura et al. 1999). In this study, we show that this localization is partially different in both human and mouse *in situ* podocytes. Having used the advanced imaging technique of STED in combination with tissue clearing, we were able to show the localization of Coro2b was not on the basal plasma membrane, where FAs connected to integrin heterodimers such as $\alpha 3\beta 1$ and others connect podocytes to the GBM (Lennon, Randles and Humphries 2014). It is quite possible that Coro2b in podocytes has a different role than it has in neuronal cells, where it was first associated with FAs. Rogg *et al*. also showed *in vitro* using a cultured immortalized podocyte cell line that Coro2b is recruited to mature FAs. A podocyte in cell culture undergoes major alterations in gene expression to reinitiate the cell cycle and proliferation (Saleem et al. 2002). Many mature *in vivo* cellular markers are downregulated or even not expressed at all. Therefore, it is possible that the role of Coro2b also is altered between *in situ* and *in vitro* podocytes. We were able to detect very low to null Coro2b expression in human immortalized podocytes. However, upon vector induced overexpression we could report the same loss of ventral F-actin fibers as Rogg *et al.* described (data not shown).

We are the first to show that the glomerular down regulation of Coro2b is restricted to DN, and does not occur to the same extent in other common nephropathies. It is somewhat surprising that the lack of Coro2b in podocytes did not affect the outcome of nephropathy caused by the STZ-induced diabetes model. The expression of Coro2b was reduced, but not significantly, in STZ-treated mice when compared to untreated wt. It might be that the Coro2b KO animals underwent genetic compensation and other proteins were taking over its tasks. Of note, the BL6 mouse strain is known to be resistant to kidney damage, as demonstrated for instance by the mild kidney disease induced by STZ treatment in these animals (Ma and Fogo 2003).

Targeting the podocyte actin cytoskeleton with PS resulted in a significant difference between KO and wt control animals in FP effacement. Already the injury model itself seemed to modulate the distribution of Coro2b in wt mice (suppl. Figure 3). PS neutralizes the anionic charge of the apical glycocalyx that maintains the FP architecture (Rudiger et al. 1999). Podocalyxin is a major transmembrane protein of the apical plasma membrane constituting a link between the intracellular actin cytoskeleton and the apical extracellular milieu (Orlando et al. 2001, Fukasawa et al. 2011). It is possible that Coro2b functions as a mediator within a signaling complex between membrane-bound podocalyxin and the actin cytoskeleton facilitating information exchange. The reaction of podocyte KOs to the PS injury and HS rescue is blunted when compared to wt animals. This could possibly partially explain the role of Coro2b in DN, where the biggest difference to other nephropathies is the constant hyperglycemic surrounding. Perhaps in response to diabetes, podocytes downregulates Coro2b in an attempt to be lessen their susceptibility to the constant stress and maintain the actin cytoskeleton integrity.

Taken together, we showed in this study that Coro2b is a podocyte-enriched protein which localizes towards the apical plasma membrane and not in basal FAs *in vivo*. We could showed that Coro2b is uniquely downregulated in DN and generated a novel podocyte specific KO mouse line to further interrogate its function. Coro2b is involved in an altered FP response in a PS and HS injury model.

4.2 THE ROLE OF CORO2B IN THE DEVELOPMENT AND FUNCTION OF THE ZEBRAFISH PRONPHROS (STUDY II)

In study II we further explored the role of Coro2b in glomerular physiology and development. Here we used the zebrafish as an alternative animal model since it is a well-established tool for early developmental *in vivo* studies. The transparent fertilized fish provide the possibility of non-invasive and easy monitoring over time.

Staining human fetal kidney sections with anti-Coro2b and anti-nephrin labeled antibodies, we determined that Coro2b expression begins at the comma-shaped glomerular body stage, which is importantly before the SD protein nephrin during human glomerulogenesis. Additionally, we confirmed with in situ hybridization and PCR of isolated pronephri from 96 h old zebrafish larvae, that Coro2b expression is conserved in zebrafish (figure 1). Using the morpholino technology, we targeted the splicing site between exon 4 and 5 of zCoro2ba and generated zCoro2ba knock-down morphants through microinjection into the freshly fertilized embryos. WB of the morphant embryos confirmed the silencing of full length zCoro2ba protein and showed the generation of a new truncated zCoro2ba protein. The knock-down of zCoro2ba did not affect the survival rate of the morphant larvae but resulted in obvious phenotypical alterations (figure 2). Sections from 96 h old zCoro2ba morphant zebrafish, embedded in JB-4 plastic and stained with Periodic acid-Schiff, revealed pronephric cysts and poorly developed pronephri with fewer cells than in controls. TEM analysis showed that the morphant podocytes exhibited FP effacement. Knock down of zCoro2ba in a podocin-GFP transgenic fish line resulted in diminished GFP signal compared to the uninjected transgenic fish, indicating decreased number of podocytes or podocyte de-differentiation (figure 3). Using an assay based on injection of high and low molecular weight fluorescence-tagged dextrans, developed to assess the filtration function of the zebrafish pronephros, we could demonstrate impaired pronephric filtration in the zCoro2ba morphants (figure 4).

It was somewhat surprising to see that in human glomerulogenesis Coro2b is already expressed during the comma-shape glomerular body stage, whereas in mice the onset of expression was reported only during the capillary loop stage of glomerulogenesis (Rogg et al. 2017). The expression pattern of Coro2b during human glomerulogenesis mimics that of nephrin with distinct staining at the developing intercellular junctions which go on to form the SD. Curiously, in our previous publication (study I) we detected Coro2b in mature podocyte FPs mainly apically and not in the basal location of the SDs. It is possible that Coro2b has a different role in the developing podocyte compared to the mature podocyte, which could also explain why in immortalized cultured podocytes Coro2b is associated with FAs. The cultured podocyte can be considered as an immature form of a podocyte, given it still retains the capability to proliferate.
The reason for the difference between the Coro2b phenotypes in our mouse and zebrafish models is unclear. One explanation could be that in the KO mice genetic compensation can occur, what in the knock down fish embryos is lacking, since at the DNA remains unimpaired and only the mRNA transcript is targeted by the morpholino. The validity of morpholino knock-downs has been questioned, as CRISPR KOs of the same genes resulted in different phenotypes compared to their respective knock-downs (Rossi et al. 2015). Since the concentration of the injected morpholino in this study was low and the survival rates were unaltered, we believe that the observed phenotype was not due to toxicity of the morpholino itself. Moreover, the impaired pronephric filtration, reduced podocin-GFP signal and FP effacement support the idea of glomerulus-specific abnormalities in

Coro2b morphants. Also, the observed pericardic edema and urinary tract distension are abnormalities that can be connected to impaired pronephros function in zebrafish larvae (Ebarasi et al. 2011, Ebarasi et al. 2009).

Taken together study I&II suggest Coro2b as an important protein for podocyte development and stress response in health and disease. Further studies to illuminate its role in more detail are assumed.

4.3 THE ROLE OF GPRC5A IN THE DEVELOPMENT OF DN (STUDY III)

The glomerulus is a major target of injury in DN and the podocyte plays an important role in its function. GPCRs constitute a large family of transmembrane receptors that are often targeted by the pharmaceutical industry because their ligands are usually small molecules and they bind extracellularly. In this study, we were looking for a podocyte enriched GPCRs to identify potential novel drug targets that could enable us to develop cell-specific therapeutic options for glomerular diseases.

By performing a high-throughput qPCR assay for GPCRs in isolated human glomeruli compared to the kidney fraction devoid of glomeruli, we identified Gprc5a as a novel glomerulus-enriched orphan GPCR previously undescribed in the kidney (suppl. table 1). Using PCR, qPCR and WB we could confirm the expression of Gprc5a in isolated glomerular fractions of man and mouse. Immunoelectron microscopy and immunohistochemistry localized Gprc5a to the glomerulus and specifically to the podocyte within the kidney cortex (figure 1). Further, we showed through semiquantitative immunohistochemistry and immunoelectron microscopic scoring of DN patient biopsies that Gprc5a is downregulated in podocytes in DN (figure 2). To assess the physiological role of Gprc5a in the kidney, we generated a Gprc5a KO mouse line using transcription activator-like effector nuclease (TALEN) technology via mutagenesis. The Gprc5a deficient mice were born in normal Mendelian distribution and exhibited no obvious phenotype at two months of age when compared with their wildtype littermates (figure 3). However, at 12 months of age, the histology of KO kidneys showed mesangial matrix expansion and elevated expression of α-smooth muscle actin (α-SMA), a marker for activation of mesangial cells. Furthermore, TEM revealed significant thickening of the GBM in 12-month old Gprc5a KO mice and upregulated expression of profibrotic factors TGF-β (35-fold), col1α1 (20-fold) and EGFR (5-fold) (figure 4). By inducing diabetes through intraperitoneal STZ injections we could show that Gprc5a KO mice developed more proteinuria, as measured by albumin/creatinine ratio, had increased mesangial expansion, signs of FSGS and totally sclerotic glomeruli. They also demonstrated increased segmental podocyte FP effacement, as shown be TEM (figure 5). Finally, we showed in immortalized cultured human podocytes that the stable overexpression of Gprc5a decreased EGFR activation, TGF-β levels, phosphorylated SMAD2/3 and col1α1 and fibronectin expression after EGF exposure (figure 6). In line with this, the silencing of Gprc5a with siRNA resulted in increased activation and expression of EGFR, TGF-β, SMAD2/3, αSMA, col1α1 and fibronectin after EGF exposure (figure 7).

In CKD fibrosis is a relevant part of the disease manifestation. EGFR and TGF-β pathways are centrally involved in the development of fibrosis. Both EGFR and TGF-β signaling are highly regulated during homeostasis and chronic dysregulation ensues severe damage. Depending on the setting, TGF-β and EGFR signaling can have adverse or beneficial effects. Therefore, it is no surprise that the systemic pharmaceutical targeting of either of the pathways has turned out to be difficult to use as an effective therapeutically option (Rayego-Mateos et al. 2018, Isaka 2018). Therefore, Gprc5a,

an effector of EGFR and TGF-β pathways, that is not ubiquitously but rather cell specifically expressed, can offer a new access point of pharmaceutical modulation. In this study we show that Gprc5a is only expressed in the podocyte in the kidney and is down regulated in DN patient biopsies. Unfortunately, it was not in the scope of this study but it would be interesting to see if the expression of Gprc5a is also differentially expressed in other common nephropathies like IgAN or MN or solely in DN. The generated Gprc5a KO mice show with age and in a diabetic STZ injury model mesangial expansion, fibrosis and thickening of the GBM phenocopying human DN glomerular pathology. Recently, Gprc5a has been shown to modulate integrin β1 (itgb1) mediated epithelial cell adhesion. CRISPR-mediated KO of Gprc5a in a panel of human epithelial cell lines correlate with reduced itgb1 expression, less activated/phosphorylated focal adhesion kinase (FAK) and diminished activity of small GTPases RhoA and Rac led to impaired cell adhesion to collagen I and IV and fibronectin (Bulanova et al. 2017). This could be a mechanism that explains the thickened GBM in our KO mice, as in podocytes integrin β1α3 plays a main role in adhesion to the subjacent GBM. Reduced adhesion and feedback signaling through lack of sufficient itgb1 might cause the podocytes to increase the production of ECM proteins like fibronectin and collagen.

In summary, Gprc5a is an interesting new podocyte-specific effector of fibrotic pathways EGFR and TGF-β and its druggability remains to be investigated but offers promise of non-systemic therapeutic intervention.

4.4 GPRC5B MODULATES INFLAMATORY RESPONSE OF GLOMERULAR DISEASE (STUDY IV)

In study IV we follow up on study III and investigate the role of Gprc5b (from the same orphan receptor subclass of GPCRs as Gprc5a) in glomerular health and disease.

In the same transcriptomic profiling of GPCRs in human glomerular and tubular fractions of study III, we also detected the glomerular enrichment of retinoic acid-induced protein 2 (RAIG2 or Gprc5b). Performing PCR, qPCR, WB and immunofluorescence microscopic co-staining with glomerular cell markers on human isolated glomeruli and cultured isolated tdtomato-expressing podocytes, we showed that Gprc5b expression is restricted to the glomeruli and the podocytes within the kidney. Immunoelectron microscopy provided further insights and located Gprc5b to the podocytes and mainly to the apical plasma membrane (figure 1). Analysis of different publicly available transcriptomic datasets and immunohistological staining and analysis of patient biopsies revealed that Gprc5b expression remains either unchanged (although other podocyte markers are downregulated) or Gprc5b is significantly upregulated in DN, IgAN and lupus nephritis (LN) (figure 2). We generated through Cre mediated loxP recombination a podocyte-specific Gprc5b KO mouse line in BL6 background. The KO animals exhibited no obvious phenotypic difference from their wildtype littermate controls as seen by light microscopic and electron microscopic analysis (figure 3). By analyzing our own and published transcriptome datasets we chose the lipopolysaccharide (LPS) induced nephropathy model to challenge the mice, since it showed upregulated levels of Gprc5b, thus phenocopying the molecular change observed in human glomerulopathies. Gprc5b-KO mice showed a delayed peak of proteinuria at 36h after the injection of LPS, instead of 24h for the control animals. Performing RNA sequencing on isolated glomeruli at 24 h after the injection revealed the differential expression of NF-κB pathway, including the upregulation of the five NF-κB proteins and downstream effectors like TNFα or IL-6 in the control animals when compared to the Gprc5b-KOs (figure 4).

Additionally, we saw down regulation of chemo-attractants as a downstream result of suppressed NF-κB activation in the KO glomeruli when compared to the wildtype animals in the LPS model. Further, immunofluorescent analysis presented decreased recruitment of CD45 positive leukocytes to the Gprc5b-KO glomeruli (figure 5). Over expression of Gprc5b in cultured immortalized human podocytes exhibited constitutive activation of NF-κB (p65) and elevated levels of downstream pro-inflammatory cytokines ccl2, IL-6 and M-CSF1. Similarly, Gprc5b expression promotes the activation/phosphorylation of EGFR and β-catenin (figure 6). Treatment of the stably overexpressing Gprc5b podocytes with activators and inhibitors of β-catenin or EGFR pathways did not modulate the constitutive phosphorylation of p65, indicating Gprc5b modulates NF-κB independent of EGFR or β-catenin activation.

In study IV we showed Gprc5b expression in the kidney to be restricted to the podocytes in mouse and human. Furthermore, we demonstrated through RNA sequencing, cell culture experiments and LPS induced nephropathy in podocyte specific Gprc5b KO mice that Gprc5b has pro-inflammatory effect in podocytes by activating NF-κB/p65 and increasing cytokine levels of ccl2 IL-6 an M-CSF1. Interestingly, Gprc5a from the same protein family of RAIGs exhibits anti-inflammatory properties by modulating EGFR-signaling and inhibiting NF-κB as described in study III. Gprc5b on the other hand has previously been associated with pro-inflammatory effects in adipocytes via modulating NF-κB. Gprc5b deficient mice appeared to be protected from diet induced obesity and insulin resistance due to suppressed inflammation in their white adipose tissue (Kim et al. 2012). Moreover, TNFα and LPS stimulated neonatal rat cardiac fibroblasts (NRCF) showed TGF-β independent upregulation of Gprc5b (von Samson-Himmelstjerna et al. 2019). In summary Gprc5b seems to have a pathogenic role in disease development in a wide variety of cell types. Finally, a study with Madin-Darby canine kidney (MDCK) cell cysts in 3D culture ascribed exosomally packed Gprc5b to have regenerative and developmental roles in tubulogenesis. Additionally, they detected exosomally packed Gprc5b in the urine of acute kidney injury (AKI) patients, proposing Gprc5b as a potential marker for AKI (Kwon, Liu and Mostov 2014).

4.5 THE ROLE OF ZFYVE28 IN PODOCYTIC EGFR-SIGNALING (STUDY V)

In an earlier microarray profiling, we discovered ZFYVE28 to be enriched in the glomerular transcriptome (Takemoto et al. 2006). As ZFYVE28 has been described to modulate EGFR signaling in *C.elegans* and mammals, we decided to investigate its role within the glomerular biology and disease development (Yoo et al. 2004, Mosesson et al. 2009).

First, we experimentally confirmed the upregulation of ZFYVE28 in the glomerulus and podocytes of mouse (6-fold) and man (42-fold) compared to the rest of the kidney by performing PCR and qPCR on isolated human and mouse glomeruli and FACS sorted tdtomato-expressing mouse podocytes. Immunofluorescence co-labeling of human kidney sections with ZFYVE28 and nephrin or vimentin localized ZFYVE28 to the podocyte FPs (figure 1). During human glomerulogenesis ZFYVE28 is earliest expressed during the early capillary stage and localizes in vicinity of nephrin at the basal aspects of pre-podocytes (figure 2). To investigate the functional role of ZFYVE28 in podocytes we generated a stably over-expressing human podocyte cell line. The ZFYVE28 over expressing podocytes showed on WB enhanced levels of activated/phosphorylated EGFR and SMAD2 after EGF stimulation, though the ratio between un-phosphorylated and phosphorylated EGFR remained similar to the control podocyte line. Confocal microscopy revealed the redistribution of EGFR from cytosolic vesicles in control podocytes to the plasma membrane in ZFYVE28 overexpressing podocytes (figure

3). To investigate the physiological role of ZFYVE28 we generated a constitutive and a podocyte-specific KO mouse line. Both KO lines were born in normal Mendelian distribution and showed no obvious phenotypical difference to the wildtype control animals in health and in an anti-GBM glomerulonephritis model (figure 4, 5, 6).

In study V we showed in extensive expressional profiling that ZFYVE28 is a novel podocyte enriched protein localizing within the FPs and its stable overexpression in cultured human podocytes enhances EGFR and SMAD2 activation and redistribution. Whereas the ZFYVE28 constitutive and podocyte specific KO animals showed no developmental or phenotype alteration from wildtype littermates in health and stress. It is possible that ZFYVE28 gets genetically compensated by another FYVE-domain containing protein. Moreover, the EGFR signaling is a multifactorial pathway structure and regulated by more than just one protein and it may be that another string within the signaling cascade takes over ZFYVE28's role. In conclusion, ZFYVE28 does not seem to be necessary for the development or maintenance of the glomerular filtration in mice.

5 CONCLUSIONS AND OUTLOOK

In this thesis we aimed to gain insights into the molecular machinery of a unique epithelial cell of the renal glomerulus, the podocyte. Its advanced state of differentiation and one-of-a-kind physiological task comes along with a specialized set of gene expression. The expertise of our lab and a collaboration with clinicians at the Karolinska University Hospital allowed us to identify and investigate podocyte enriched proteins in health and disease. Although, we generated quite a lot of new data on the novel podocyte enriched proteins, many questions remain to be asked and answered about their role and function in podocyte maintenance.

Combining the data of study I&II revealed new information about actin cytoskeleton associated protein Coro2b. We showed that it is uniquely downregulated in DN in contrast to seemingly staying reasonably unaffected in IgAN or MN. It would be interesting to analyze the expression profile of Coro2b in other nephropathies to confirm its exclusive downregulation in DN. Or perhaps it is downregulated also in another diseases and if so allowing to find a common determinant? We showed that Coro2b in podocyte FPs does not locate at the basal plasma membrane, questioning therefore the previous finding of Coro2b recruitment to FAs. If it is not involved in podocytic FAs like it is in neurite tips, how does it regulate the actin cytoskeleton in FPs? We showed that the lack of Coro2b affects podocyte FP effacement and reorganization in response to PS injury and HS rescue. PS affects the actin cytoskeleton organization via apical plasma membrane protein podocalyxin. Perhaps Coro2b associates with it and further biochemical experiments could give some more insight to its role in podocytes. Although Coro2b KO mice in BL6 background failed to demonstrate an obvious renal phenotype, we could show that the morpholino mediated knock down of Coro2ba in zebrafish embryos leads to decreased number of podocytes, podocyte FP effacement and impaired pronephric filtration barrier. Obviously, further experiments to investigate the reason for the phenotype difference are needed. Does in the KO mouse genetic compensation occur? Would we also have no developmental effects if we generated a zebrafish KO? Or do we see no renal phenotype in BL6 mice due to their renopathy-resistant genetic background? Would Coro2b KO mice show a renal phenotype in 129Sv genetic background? To elucidate some of these questions we have already started with the generation of a Coro2ba CRISPR-KO zebrafish.

In study III we identified orphan GPCR Gprc5a as a podocyte enriched transmembrane receptor, which is downregulated in DN. Aging Gprc5a deficient mice exhibit signs reminiscent of DN histopathology, such as GBM thickening and elevated levels of mesangial cell activation marker α-SMA and profibrotic factors TGF-β, col1α1 and EGFR. In a STZ induced diabetes model Gprc5a KO develop higher levels of albuminuria, more severe mesangial matrix expansion and sclerotic glomeruli when compared to their littermate controls. Additionally, we showed that the stable overexpression of Gprc5a in cultured human podocytes inhibited the activation of EGFR, TGF-β levels, SMAD2/3 and expression of col1α1 and fibronectin, whereas siRNA mediated silencing of Gprc5a increased the activation and expression of the same proteins after EGF exposure. One obvious question is to investigate the role of Gprc5a in other glomerulopathies. EGFR-dependent signaling pathways in podocytes have been shown to be important in crescentic glomerulonephritis. To answer this, we are in the process of analyzing the expression of Gprc5a in disorders associated with crescent formation, as well as challenging our KO mice with a model of glomerulonephritis. Since Gprc5a is lacking a known ligand the search for a small molecule binding to inhibit or activate Gprc5a would be the next step to see if the modulation of EGFR-signaling through Gprc5a could work as a future drug target in DN. This is, however challenging as the down-stream signaling pathway is still poorly characterized.

Study IV describes Gprc5b as another orphan GPCR highly enriched in the podocytes. We analysed transcriptomic information and showed that Gprc5b's expression is upregulated in DN, IgAN and LN. Gprc5b has a pro-inflammatory effect in podocytes by activating NF-κB/p65 and increasing cytokine levels of ccl2 IL-6 an M-CSF1. Gprc5b KO mice used in the study were in a mixed BL6/129Sv background. It would be interesting to see if there is a different phenotype in a strain more susceptible to kidney damage, such as a pure 129Sv background. Also, using morpholinos in zebrafish embryos would give insight into Gprc5b's importance in development and glomerular/pronephric function. Since Gprc5b is upregulated in DN, LN and IgAN promoting inflammation via NF-κB, it would be of value to develop a mouse line in which Gprc5b expression can be induced in podocytes. Moreover, it would be of value to find a possible small molecule inhibitor by high-throughput screening as a possible drug compound to inhibit glomerular inflammation. Since Gprc5b and Gprc5a are from the same family of orphan GPCRs we have started to crossbreed the two KO strains to generate a double KO to see whether their effects abrogate one another or result in an enhanced disturbance of the EGFR/NF-κB signaling.

In study V we generated information about ZFYVE28 showing it is highly expressed in podocytes of man and mouse. Cultured human podocytes overexpressing ZFYVE28 exhibit enhanced levels of activated EGFR and SMAD2 after stimulation with EGF. KO of ZFYVE28 in BL6 mice does not affect the phenotype in health or disease. Possibly, we have not used the right injury model or the BL6 strain is too resistant against kidney injury to have developed a phenotype investigating this could proof ZFYVE28 of importance in podocyte homeostasis.

To conclude, we investigated in this thesis different novel podocyte proteins and gave insight into their respective roles in podocyte development, homeostasis or stress response. Many investigated proteins are differentially expressed in human kidney disease and some even modulate the glomerular response to injury. Therefore, further studies on their suitability as potential drug targets are indicated.

6 POPULAR SCIENCE SUMMARY

The kidneys are the organ that have an important task to filter the blood from harmful waste products and get rid of them through the urine they create. A person has usually two kidneys but in rare cases people are born with just one kidney or even a "super-kidney", called horseshoe kidney due to its visual likeliness of the two kidneys joining across the midst of the body center, forming one. But how do the kidneys manage to filter blood so that only the waste products get flushed out with the urine and not important nutrients? Initially they don't - the primary selection is by mainly by size, proteins smaller than blood plasma transport protein albumin will get filtrated into the primary urine and only on its way through the kidney to the bladder urine gets formed, as important nutrients, salts and vitamins get reabsorbed into the bloodstream. This process is managed by the functional unit of the kidney termed the nephron. At the head of the nephron is the glomerulus where the blood vessels split up into a mesh of tiny capillaries with specialized endothelial cell walls that allow liquid to pass through. Surrounding, the "leaky" capillaries are cells of even further specialization, the podocytes. The podocytes (podo=feet; cyte=cell) were named due to their unique cell shape, which looks a bit like an octopus. They have one major cell body with the nucleus or cell core and from that body multiple protrusions extend like branches would off a tree trunk. Each of those branches, like on a tree, has further smaller twigs or in case of the podocyte: foot processes branching off. The foot processes of neighboring podocytes intertwine and form a sieve hindering the blood contents to pass freely. The "leaky" endothelium, the podocytes and a collagenous layer between the two form the filtration barrier where the blood gets filtered into the surrounding space, called Bowman's capsule as primary urine. From there on the primary urine passes through a winding pipe system, the tubuli, where water, minerals, salts and nutrients get reabsorbed into the bloodstream before the filtrate arrives as urine in the ureter and the bladder. In kidney disease one of the first changes detected is in the glomerulus where the filtration happens, seen as the loss of ordinary podocyte foot process structure. Damage to podocyte cells has been shown to be important for the progression of many kidney diseases

Kidney disease or kidney failure is a worldwide health problem and the main causes are diabetes and high blood pressure. There are little options of treatment and so far those only slow down the progression of the disease before it ends in kidney failure or medical termed end stage renal disease (ESRD). Patients with ESRD are faced with a lifelong sentence, which might not be much, of dialysis treatments up to 5 times per week unless they are eventually lucky enough to receive a matching donor kidney. Therefore, in this thesis we aimed to find and study proteins that make the podocytes so different from other cells. We speculated that if we can identify and target such unique podocyte proteins pharmaceutically early on to make podocytes more resistant to damage, we could develp a new therapeutical option to slow kidney diseases from progressing into ESRD.

In study I&II we showed that Coro2b is a podocyte protein, which is only down regulated in diabetic kidney disease and not in other common kidney diseases. In experiments where we deleted the protein sequence from the DNA to generate knock out (KO) mice or stopped the protein formation of Coro2b in zebrafish eggs/embryos showed us that Coro2b can be involved in the development of podocytes and their reaction to injury.

In study III&IV we identified two receptor proteins from the same class, Gprc5a and Gprc5b that sit in the cell membrane/wall of podocytes, enabling them to transfer signals from intra-to extracellular and backwards. We showed with KO mice and cell culture experiments that they both modulate pathways

regulating inflammation and fibrosis, NF-κB and EGFR pathway, but in an opposite way. Gprc5a is anti-fibrotic, whereas Gprc5b is profibrotic. We also showed that in human kidney diseases Gprc5a is downregulated and Gprc5b is upregulated.

In study V we demonstrated that the protein ZFYVE28 is expressed in the podocytes and shows pro-fibrotic properties in cell culture experiments. In an animal model we did not see any effect in health or disease when ZFYVE28 was knocked out.

Coro2b, Gprc5a and Gprc5b are affected in human kidney diseases and we showed in different experiments their involvement in disease development and podocyte well-being, indicating that further studies are needed to test them for their suitability as drug targets in the battle against the progression of kidney disease.

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